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Independent function of two destruction domains in hypoxia-inducible factor-α chains activated by prolyl hydroxylation

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Oxygen-dependent proteolytic destruction of hypoxia-inducible factor-α (HIF-α) subunits plays a central role in regulating transcriptional responses to hypoxia. Recent studies have defined a key function for the von Hippel–Lindau tumour suppressor E3 ubiquitin ligase (VHLE3) in this process, and have defined an interaction with HIF-1α that is regulated by prolyl hydroxylation. Here we show that two independent regions within the HIF-α oxygen-dependent degradation domain (ODDD) are targeted for ubiquitylation by VHLE3 in a manner dependent upon prolyl hydroxylation. In a series of *in vitro* and *in vivo* assays, we demonstrate the independent and non-redundant operation of each site in regulation of the HIF system. Both sites contain a common core motif, but differ both in overall sequence and in the conditions under which they bind to the VHLE3 ligase complex. The definition of two independent destruction domains implicates a more complex system of pVHL–HIF-α interactions, but reinforces the role of prolyl hydroxylation as an oxygen-dependent destruction signal.

Keywords: destruction domain/hypoxia-inducible factor-α/prolyl hydroxylation/ubiquitylation/ von Hippel–Lindau protein

Introduction

The hypoxia-inducible factor (HIF) transcriptional system plays a central role in physiological responses to oxygen availability, and regulates genes involved in processes such as angiogenesis, erythropoiesis, vasomotor control, energy metabolism, carbon dioxide metabolism and cell survival decisions (Semenza, 2000; Wenger, 2000). Although oxygen availability can influence multiple steps in HIF activation (Jiang *et al*., 1997; Pugh *et al*., 1997; Huang *et al*., 1998; Kallio *et al*., 1998; Bhattacharya *et al*., 1999), the primary mode of regulation occurs through oxygen-dependent proteolysis of HIF-α subunits (Huang *et al*., 1996, 1998; Pugh *et al*., 1997; Salceda and Caro, 1997). In normoxic cells, HIF-α subunits have an exceptionally short half-life (Jewell *et al*., 2001), and steady-state levels are very low. Increasing severity of hypoxia retards degradation of HIF-α subunits in a graded manner (Jiang *et al*., 1996), allowing nuclear localization, dimerization with HIF-β and formation of a DNA-binding HIF complex.

HIF-α subunits are therefore part of a large set of cellular regulators whose activity is determined by tightly controlled proteolysis. Many of these molecules are known to contain transferable destruction domains that can confer instability on heterologous proteins. In some cases, phosphorylation of particular residues provides a specific recognition signal that targets the substrate to ubiquitin ligase complexes, though in many cases the determinants that regulate proteolysis remain unknown (Jackson *et al*., 2000).

In the case of HIF, two isoforms of the α-subunit, HIF-1α and HIF-2α, have been shown to be regulated in a similar manner. Each possesses an extensive, transferable, oxygen-dependent degradation domain (ODDD) encompassing >200 residues in the central region of the molecule (Huang *et al*., 1998; Ema *et al*., 1999; O'Rourke *et al*., 1999; Sutter *et al*., 2000). Interestingly studies of isolated sequences have demonstrated that partial instability can be conveyed by subdomains within the ODDD (Huang *et al*., 1998; O'Rourke *et al*., 1999; Yu *et al*., 2001). Since these domains must interact with the oxygen-sensitive signal, their sequences have been analysed intensively in an effort to gain an understanding of the sensing/transduction process. Important insights have been gained from studies of interactions of HIF-α with the von Hippel–Lindau tumour suppressor protein (pVHL) (Maxwell *et al*., 1999). pVHL is part of a multiprotein ubiquitin E3 ligase complex (VHLE3), homologous to the SCF (Skp-1-Cdc53/Cullin-F-box) class of E3 ligases (Lisztwan *et al*., 1999; Stebbins *et al*., 1999). pVHL itself plays a role analagous to the F-box substrate recognition component, and can interact directly with HIF-α subunits and target them for VHLE3-dependent ubiquitylation *in vitro* (Cockman *et al*., 2000; Kamura *et al*., 2000; Ohh *et al*., 2000). Protein interaction and ubiquitylation assays have defined a subdomain in the C-terminal portion of the HIF-1α ODDD that is necessary and sufficient for VHLE3-dependent ubiquitylation under the conditions of assay, and shown that residues 556–574 of HIF-1α constitute a minimal pVHL-binding domain within this region (Cockman *et al*., 2000; Ohh *et al*., 2000; Tanimoto *et al*., 2000). Interaction of this region with pVHL is promoted by enzymatic hydroxylation of Pro564 (Ivan *et al*., 2001; Jaakkola *et al*., 2001). Although the HIF-prolyl hydroxylase(s) remain to be characterized, these findings are of interest in relation to the mechanism of oxygen sensing. Since known enzymes of this class are dioxygenases that utilize molecular oxygen as co-substrate (Kivirikko and Myllyharju, 1998), these findings provide a direct link between the availability of oxygen and the regulation of HIF. In the simplest model, the availability of oxygen would affect the rate of modification of Pro564

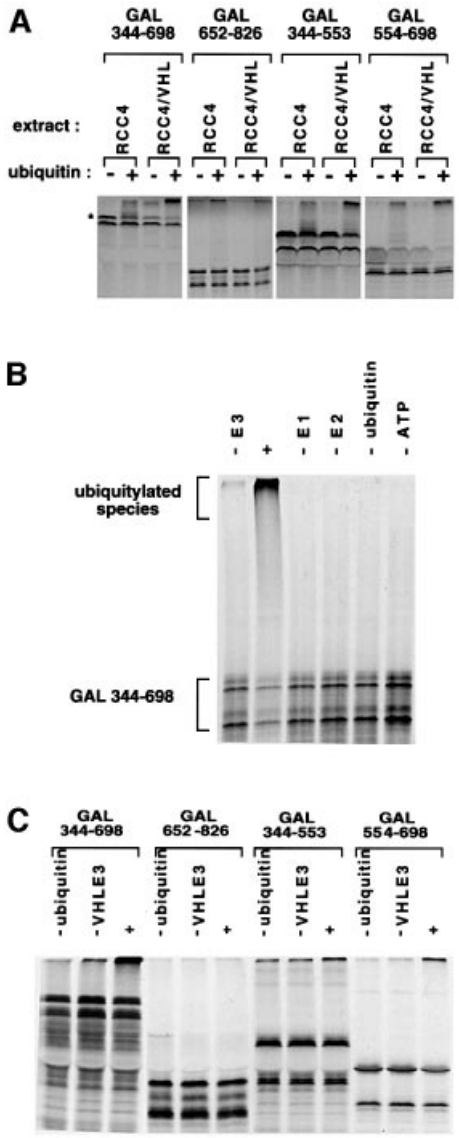

Fig. 1. The VHLE3 ligase can interact functionally with two distinct regions of the HIF-1α ODDD. (**A**) Ubiquitylation of [^{35}S]methionine-labelled GAL–HIF-1α fusion proteins by cytoplasmic extracts from VHL-defective RCC4 cells, or RCC4 cells stably transfected with pcDNA3-VHL (RCC4/VHL). Reactions were performed in the presence or absence of exogenous ubiquitin as indicated. VHLE3-dependent ubiquitylation, resulting in a strong signal of decreased mobility at the top of the lane, is seen when the substrate contained HIF-1α amino acids 344–698, 344–553 and 554–698, but not amino acids 652–826. The asterisk denotes the full-length, correctly initiated GAL344–698 fusion protein (see Materials and methods). (**B**) VHLE3-dependent ubiquitylation by purified components of the pathway. Ubiquitylation of an immunopurified GAL–HIF-1α fusion protein (GAL344–698) occurred in the presence of E1, E2, VHLE3 (E3), ubiquitin and ATP (lane marked '+') but not in the absence of any individual component. (**C**) Ubiquitylation of different GAL–HIF-1α fusion proteins in the purified component assay. Reactions were performed using all components (+), or mixtures lacking either ubiquitin (–ubiquitin) or VHLE3 (–VHLE3). VHLE3-dependent ubiquitylation is seen when the substrate contained HIF-1α amino acids 344–698, 344–553 and 554–698, but not amino acids 652–826.

and hence degradation of HIF-α by the VHLE3–ubiquitin–proteasome pathway. However, such a model provides little insight into how the complex physiological response to oxygen availability is controlled with such precision, does not define a role for the majority of the HIF-α ODDD sequences and cannot explain the partial instability conveyed by other regions of the ODDD.

To investigate this, we have analysed interactions between HIF-α ODDDs and the VHLE3 complex using a series of ubiquitylation and interaction assays based on crude cell lysates or purified components of the ubiquitylation system. We show that two independent regions of the HIF-1α ODDD are targeted for ubiquitylation by VHLE3 in a manner that is dependent upon hydroxylation of specific proline residues. Although both proline residues are located in a motif that is conserved between HIF-1α and HIF-2α at both sites, the target sites differ in overall sequence and requirements for interaction with pVHL.

Results

The VHLE3 ligase can interact functionally with two distinct regions of the HIF-1α ODDD

The VHLE3-dependent ubiquitylation of different portions of the HIF-1α ODDD was first assayed in an *in vitro* system using cytoplasmic extracts from VHL-defective renal carcinoma (RCC4) cells and stable transfectants re-expressing pVHL (RCC4/VHL). [^{35}S]methionine-labelled HIF-1α ODDD sequences were expressed as GAL4 fusions in coupled *in vitro* transcription/translation (IVTT) reactions in rabbit reticulocyte lysate, then added to the ubiquitylation reactions. As anticipated, GAL344–698 (encompassing the entire ODDD), but not GAL652–826 (encompassing the stable HIF-1α C-terminus), manifested VHLE3-dependent ubiquitylation (Figure 1A). Some non-VHLE3-dependent ubiquitylation was observed in these assays but is of uncertain significance since it was not related specifically to the presence of ODDD sequences. Comparison of the entire HIF-1α ODDD with a C-terminal subdomain demonstrated more efficient ubiquitylation with the full-length ODDD (compare GAL344–698 and GAL554–698, Figure 1A). We therefore tested the N-terminal subdomain of the HIF-1α ODDD (GAL344–553) in isolation, and found that this also supported VHLE3-dependent ubiquitylation, implying that it too could interact with the VHLE3 complex. In view of previous analyses of interactions between pVHL and HIF-1α, this result was somewhat surprising. These analyses have implicated only one domain at the C-terminus of the HIF-1α ODDD (residues 556–574) as capable of interaction with pVHL (Cockman *et al*., 2000; Ohh *et al*., 2000; Tanimoto *et al*., 2000), and shown that this interaction is dependent on enzymatic hydroxylation of HIF-1α residue Pro564 by an activity that is present in the reticulocyte lysate used for IVTT (Ivan *et al*., 2001; Jaakkola *et al*., 2001). In an effort to understand the current results, we considered that the RCC4 cytoplasmic extract used in the ubiquitylation assays might contain additional activities that were not present in the reticulocyte lysate, and that were required to promote functional interactions between other portions of the HIF-1α ODDD and VHLE3.

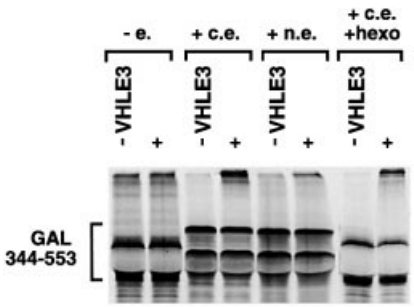

Fig. 2. Effect of pre-incubation of GAL344–553 substrate with cell extracts on VHLE3-dependent ubiquitylation. The substrate was incubated in buffer alone (–e.), or in cytoplasmic extract (+c.e.), nuclear extract (+n.e.) or cytoplasmic extract that had been depleted of ATP by incubation with hexokinase and glucose (+c.e. +hexo). All extracts were prepared from RCC4 cells lacking pVHL. Following immunopurification, the GAL344–553 substrates were ubiquitylated in the purified component assay in the presence (+) or absence (–VHLE3) of VHLE3. Pre-treatment with cytoplasmic extract greatly enhanced VHLE3-dependent ubiquitylation.

Cytoplasmic extract enhances functional interaction of the VHLE3 ligase with an N-terminal target site in HIF-1α

To permit separate analysis of cytoplasmic activities that might promote functional interactions with VHLE3, distinct from the ubiquitylation activity itself, we developed a purified component assay for VHLE3-dependent ubiquitylation (see Materials and methods). Addition of GAL344–698 substrate to this assay resulted in the ubiquitin- and ATP-dependent production of high molecular weight species that correspond to ubiquitylated forms of GAL344–698. Omission of different components from the assay indicated that production is E1, E2 and VHLE3 dependent (Figure 1B). Testing of HIF-1α domains as GAL fusions in this assay demonstrated that whilst GAL652–826 was not ubiquitylated, both GAL344–553 and GAL554–698 were targets for the VHLE3 ligase (Figure 1C). However, VHLE3-dependent ubiquitylation of the two portions of the HIF-1α ODDD differed markedly from that in the assays based on crude cytoplasmic extracts. In the assay using cytoplasmic extract, GAL344–553 was at least as good a substrate for VHLE3-dependent ubiquitylation as GAL554–698 (Figure 1A), but in the assay using purified components, GAL344–553 was a poorer substrate (Figure 1C). To test whether an activity in the cytoplasmic extract was responsible for this difference, GAL344–553 substrate was pre-incubated with either buffer, cytoplasmic extract or nuclear extract, then immunoprecipitated and assayed for VHLE3-dependent ubiquitylation (Figure 2). The buffer-treated substrate remained a poor target for VHLE3, but pre-treatment with cytoplasmic extract was found to have a striking effect, converting the GAL344–553 substrate into a much better target for VHLE3-dependent ubiquitylation (Figure 2). Although the cytoplasmic extract used was derived from VHL-defective RCC4 cells, cytoplasmic extract from other tissue culture cell types was found to have the same effect (data not shown). These results therefore confirmed the existence of a VHL interaction site in the N-terminal portion of the HIF-1α ODDD, and indicated that exposure of the HIF-1α polypeptide to a factor present in cytoplasmic but not reticulocyte extract was important for this function.

Interestingly, pre-treatment with cytoplasmic extract resulted in a marked mobility shift of GAL344–553 (Figure 2). HIF-1α mobility previously has been demonstrated to be affected by phosphorylation both *in vitro* and *in vivo* (Richard *et al*., 1999; Berra *et al*., 2000), although this has not been localized to a specific region of the protein. Since substrate phosphorylation regulates interactions with other E3 ligase complexes (Patton *et al*., 1998), a potential link between phosphorylation and ubiquitylation of this region was therefore considered. However, pre-incubation of the GAL344–553 substrate with nuclear extract also resulted in a mobility shift, that was not accompanied by increased VHLE3-dependent ubiquitylation (Figure 2). Furthermore, cytoplasmic extracts that were depleted of endogenous ATP by pre-incubation with hexokinase no longer facilitated the GAL344–553 mobility shift, but still promoted enhanced VHLE3-dependent ubiquitylation (Figure 2), indicating that ATP-dependent modifications of GAL344–553 (including phosphorylation) are not the event mediating interaction with VHLE3.

Mapping of 380–417 as a minimal domain targeted by cytoplasmic extract and VHLE3

To analyse the interaction between pVHL and the N-terminal HIF-1α ODDD sequences further, we sought to define a minimal functional domain in this region that would support VHLE3-dependent ubiquitylation. Resi-Iyer *et al*., 1998). Deletion of individual exons localized the activity to exon 9, represented by GAL344–417 (Figure 3A). Testing of the corresponding exon in HIF-2α (residues 345–416) (Tian *et al*., 1997) indicated that despite limited conservation, this sequence was also a target for VHLE3-dependent ubiquitylation following cytoplasmic extract pre-treatment (Figure 3B).

Further deletional analysis of HIF-1α sequences defined a minimal domain of residues 360–417 that was necessary for maximal ubiquitylation. Deletion to residues 380–417 greatly reduced ubiquitylation, although some activity was still observed (Figure 3C).

However, when tested in an interaction assay with VHLE3, GAL380–417 bound to VHLE3 as efficiently as GAL344–553, and binding showed similar dependence on pre-treatment with cytoplasmic extract (Figure 3D). Further deletions ablated (GAL380–410) or markedly lowered (GAL390–417) binding to the VHLE3 complex. Thus, these experiments indicated that residues 380–417 are sufficient to support both modification by cytoplasmic extract and interaction with VHLE3, but that additional sequences are required for efficient ubiquitylation.

Identification of a potential core motif conserved between the N- and C-terminal VHLE3 target sites

The HIF-1α 380–417 sequence was analysed further to define functionally critical residues. Alignment with the corresponding region of HIF-2α and the known VHL-binding site in the C-terminal region of the HIF-1α ODDD (Figure 4A) revealed a common motif, LXXLAP, which was conserved between species. Mutations of this motif were assayed in the context of GAL344–417 (Figure 4B). The double mutation of Leu397 and Leu400 to alanine

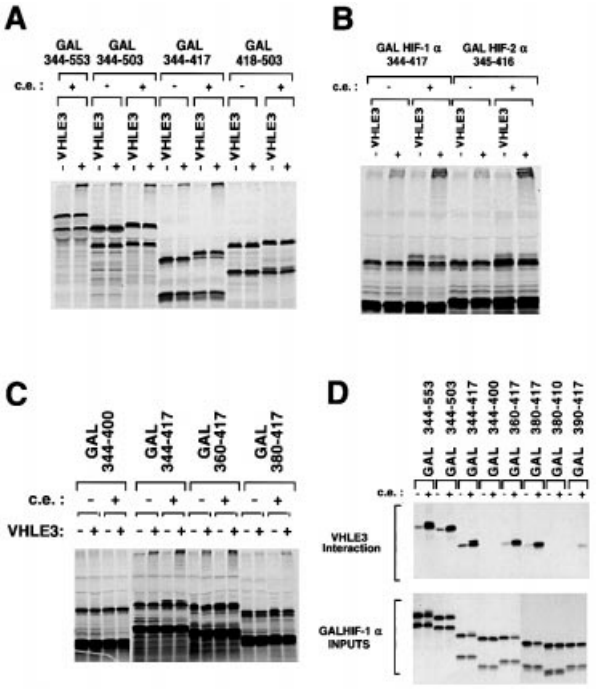

Fig. 3. Determination of HIF-α sequences that support VHLE3 interaction and ubiquitylation. (A–C) Ubiquitylation of GAL-HIF-1α or GAL–HIF-2α fusion protein substrates that had been pre-incubated in buffer alone (–c.e.) or in cytoplasmic extract (+c.e.), immunopurified and then added to reaction mixes either containing (+) or lacking VHLE3 (–VHLE3). (**A**) Exon-based analysis demonstrating that sequences encoded by HIF-1α exon 9 (residues 344–417) constitute an N-terminal ODDD target site for VHLE3-dependent ubiquitylation. Note that the actual exon 10–11 boundary is at amino acids 512–513. (**B**) Assay of homologous HIF-1α and HIF-2α sequences indicating that this VHLE3 target site is conserved between the HIF-α isoforms. (**C**) Further analysis of the N-terminal ODDD target site indicating that HIF-1α residues 360–417 are required for efficient ubiquitylation, whereas residues 380–417 support reduced but still significant VHLE3-dependent ubiquitylation. (**D**) VHLE3 interaction assay. The indicated [^{35}S]methionine-labelled GAL–HIF-1α fusion proteins were incubated either in buffer alone (–c.e.) or in cytoplasmic extract (+c.e.). 786-0 HA·VHL cell extract (prepared in buffer that does not support VHLE3 target site modification) was then added and anti-HA immunoprecipitation performed. The retrieved immunoprecipitates and input samples of the GAL–HIF-1α fusion proteins were analysed by SDS–PAGE and autoradiography. HIF-1α residues 380–417 constitute a minimal domain capable of interaction with VHLE3 after exposure to c.e.

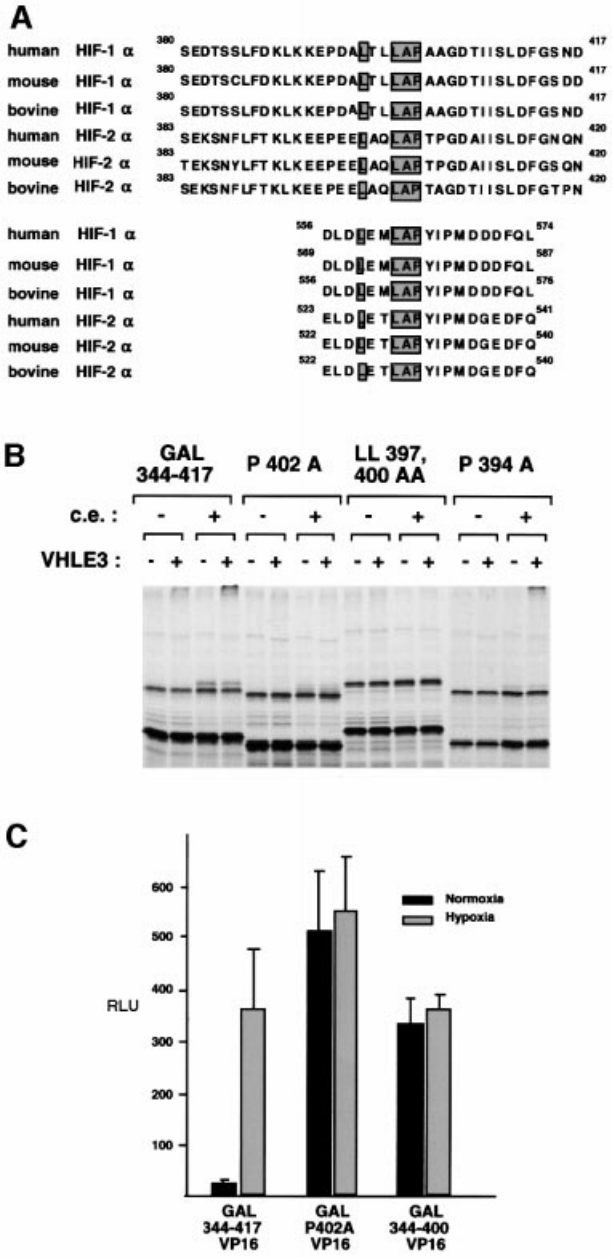

Fig. 4. Identification of a potential core motif targeted by VHLE3. (**A**) The amino acid sequences of the N- and C-terminal VHLE3-binding sites identified in human HIF-1α are aligned with the corresponding regions in other species (as indicated) and with HIF-2α sequences. A core motif is shaded. (**B**) Effect of mutations in this core motif on VHLE3-dependent ubiquitylation. The GAL344–417 substrate and the indicated mutant derivatives were pre-treated with buffer alone (–) or with cytoplasmic extract (+), immunopurified then added to ubiquitylation reactions that did (+) or did not (–) contain VHLE3. Mutants P402A and LL397,400AA, but not P394A, ablate activity. (**C**) Oxygen-regulated activity of GAL–HIF-1α–VP16 fusion proteins containing the indicated HIF-1α sequences, in U2OS cells co-transfected with the GAL reporter pUAS-tk-Luc. Columns show corrected luciferase activity, mean ± 1 SE of three independent experiments. Regulated fusion protein activity is observed with HIF-1α residues 344–417, but not a P402A mutant derivative, or HIF-1α residues 344–400.

(LL397, 400AA) was found to ablate VHLE3-dependent ubiquitylation (Figure 4B). Point mutation of Pro402 to alanine also ablated VHLE3-dependent ubiquitylation (P402A, Figure 4B). In contrast, mutation of the second proline residue in the 380–417 region had no effect (P394A, Figure 4B).

Mutations of the 344–417 region were then tested for their effects on oxygen-dependent responses *in vivo*. HIF-1α residues 344–417 are known to confer oxygen-dependent regulation on the activity of a GAL–VP16 fusion protein in transiently transfected cells (O'Rourke *et al.*, 1999). Both the C-terminal deletion (344–400) and the P402A mutation were tested in this context, and were each found to abolish regulation of GAL–VP16 fusion protein activity by oxygen (Figure 4C).

Identification of critical point mutations allowed these two VHLE3 target sites to be assayed within the full-length HIF-1α molecule. The P402A mutation was introduced to ablate activity of the N-terminal VHLE3 target site, and the previously described P564G mutation (Jaakkola *et al.*, 2001) was introduced to ablate activity of

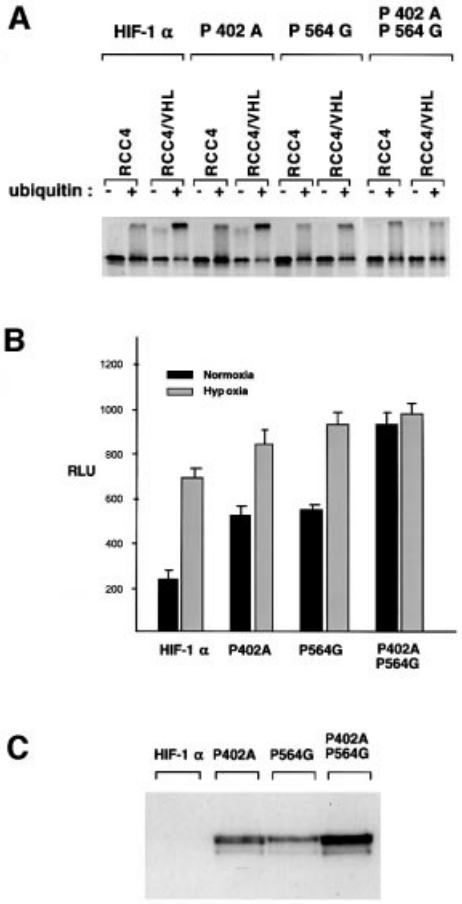

Fig. 5. Proline residues 402 and 564 are critical for HIF-1α regulation both *in vitro* and *in vivo*. (**A**) Ubiquitylation of wild-type HIF-1α or the indicated mutants by cytoplasmic extracts from RCC4 or RCC4/VHL cells in the presence (+) or absence (–) of exogenous ubiquitin. The double mutant P402A + P564G shows no VHLE3-dependent ubiquitylation, but single mutations of the critical proline residues at each individual VHLE3 target site only partially reduce ubiquitylation. (**B**) Activity of transiently transfected wild-type HIF-1α and the indicated mutant derivatives, in HIF-1α-deficient CHO-Ka13 cells co-transfected with the HRE reporter plasmid pGL3PGK6TKp. Columns show corrected luciferase activity, mean ± 1 SE of three independent experiments. The single proline mutants show partially enhanced normoxic activity compared with the wild-type HIF-1α, whereas the combined mutant showed full constitutive activity in normoxia. (**C**) Immunoblot analysis of transiently transfected wild-type HIF-1α and the indicated mutant derivatives, in extracts from HIF-1α-deficient CHO-Ka13 cells grown under normoxic conditions.

the C-terminal VHLE3 target site. When these mutations are introduced individually, the mutant HIF-1α proteins still remained targets for VHLE3-dependent ubiquitylation, presumably because each retains an active VHLE3 target site (Figure 5A). However, a double mutant showed no VHLE3-dependent ubiquitylation at all (P402A + P564G, Figure 5A). HIF-1α therefore appears to contain two and only two discrete target sites for VHLE3-dependent ubiquitylation. To test the functional importance of these sites *in vivo*, HIF-1α molecules bearing the single and double VHLE3 target site mutants were transfected into the HIF-1α-deficient cell line Ka13 (Wood *et al*., 1998) and tested for their ability to mediate oxygen-dependent transcriptional regulation (Figure 5B). When compared with wild-type HIF-1α, P402A and P564G mutants were more active under normoxic conditions and showed reduced up-regulation in hypoxia, whereas the P402A + P564G double mutant had constitutive activity under normoxic conditions (Figure 5B). To confirm that the differences in reporter gene regulation were due to differences in HIF-1α protein stability, immunoblot analysis was performed on extracts from transfected Ka13 cells grown under normoxic conditions (Figure 5C).

The N-terminal VHLE3 target site is regulated by prolyl hydroxylation

The critical dependence of N-terminal ODDD function on a proline residue within a conserved motif, together with dependence on prior incubation with a cytoplasmic extract, suggested that this site might represent a second site of modification by enzymatic prolyl hydroxylation. In keeping with this, we found that the activity in the cytoplasmic extract was heat labile (data not shown) and that the addition of the prolyl hydroxylase cofactor iron (II) augmented the ability of cytoplasmic extract to promote ubiquitylation of the isolated N-terminal sequence GAL344–417 in a manner similar to the effect on the C-terminal site (GAL554–698) (Figure 6A).

To assess the potential role of enzymatic prolyl hydroxylation further, GAL–HIF-1α substrates were synthesized *in vitro* in the presence of the proline analogue 3,4-dehydro-L-proline. Incorporation of this analogue into substrates renders them refractory to prolyl hydroxylation (Rosenbloom and Prockop, 1970). When synthesized in this way, both the N- and C-terminal VHLE3 target sites (represented by GAL344–553 and GAL554–698, respectively) were unable to interact with VHLE3 (Figure 6B). A more specific effect of 3,4-dehydro-L-proline on the N-terminal site was achieved using a GAL380–417 substrate in which the proline residue at position 394 was mutated to alanine (GAL380–417 P394A). In this substrate, the only HIF-1α proline residue is at position 402. When synthesized in the presence of 3,4-dehydro-L-proline, GAL380–417 P394A was also incapable of interaction with VHLE3 (Figure 6B), strongly suggesting that hydroxylation at Pro402 may be critical for promotion of the VHLE3 interaction. Further evidence was provided by the effect of *N*-oxalylglycine, a 2-oxoglutarate analogue that acts as a competitive inhibitor of prolyl hydroxylases and which previously has been shown to inhibit hydroxylation of HIF-1α Pro564 (Jaakkola *et al*., 2001). Modification of GAL380–417 P394A was inhibited by *N*-oxalylglycine but could be restored partially by 2-oxoglutarate (Figure 6C). To test the role of prolyl hydroxylation directly, we synthesized N-terminal biotinylated peptides corresponding to HIF-1α residues 390–417 that contained either proline (B28PRO) or hydroxyproline (B28HYP) at residue 402, and tested for the effect of hydroxyproline substitution in binding and ubiquitylation assays. B28HYP but not B28PRO was able to co-precipitate the VHLE3 complex (Figure 6D). Furthermore, when added to the ubiquitylation reaction, B28HYP but not B28WT peptide specifically blocked ubiquitylation of GAL344–553 (Figure 6E). In keeping with the deletional analysis of this region, B28PRO could not be modified by cytoplasmic extract so as to capture VHLE3 (Figure 6D). Thus, the data would be consistent

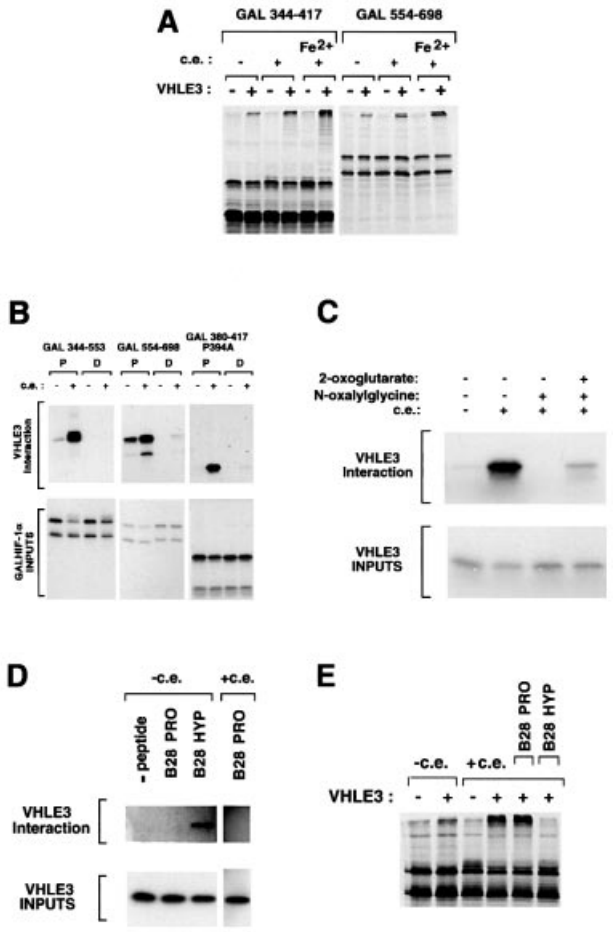

Fig. 6. The N-terminal VHLE3 target site is regulated by prolyl hydroxylation. (**A**) Effect of iron (II) supplementation on VHLE3-dependent ubiquitylation. GAL344–417 and GAL554–698 substrates were pre-incubated in buffer alone (–), in cytoplasmic extract (+) or in cytoplasmic extract with added iron (Fe^{2+}+). Following immunopurification, substrates were ubiquitylated in the purified component assay in the presence (+) or absence (–) of VHLE3. Both substrates exhibit VHLE3-dependent ubiquitylation, which is enhanced by pre-treatment with additional iron. (**B**) Effect of the proline analogue 3,4-dehydro-L-proline on VHLE3 interaction. [^{35}S]methionine-labelled GAL–HIF-1α substrates were prepared in reactions containing excess L-proline (P) or 3,4-dehydro-L-proline (D), and tested for VHLE3 interaction using 786-0 HA·VHL cell extract as in Figure 3D (see Materials and methods). Autoradiographs show SDS–PAGE analyses of the interacting GAL–HIF-1α proteins in anti-HA immunoprecipitates, together with the input samples. (**C**) Effect of the prolyl hydroxylase inhibitor *N*-oxalylglycine on modification of GAL380–417 P394A substrate. *N*-oxalylglycine (1 mM) inhibited the modifying activity of cytoplasmic extract and prevented VHLE3 interaction. Modification was partially restored by the addition of 5 mM 2-oxoglutarate. (**D**) Interaction of VHLE3 with synthetic peptides. Biotinylated peptides corresponding to HIF-1α residues 390–417 (B28PRO), or the corresponding peptide with Pro402 substituted with hydroxyproline (B28HYP), were incubated with buffer alone (–c.e.) prior to incubation with 786-0 HA·VHL cell extract. Following retrieval of peptide using streptavidin beads, captured HA·VHL was detected by anti-HA immunoblotting. B28HYP but not B28PRO captured HA·VHL. B28PRO was also incubated with cytoplasmic extract (+c.e.) prior to VHLE3 interaction. (**E**) Excess B28HYP but not B28PRO blocks VHLE3-dependent ubiquitylation. GAL344–553 substrate was pre-treated with buffer alone (–c.e.) or with cytoplasmic extract (+c.e.), then added to the purified component ubiquitylation assay in the presence or absence of peptide as indicated.

with a model in which HIF-1α residues 390–417 provide a discrete interaction site for VHLE3 upon hydroxylation of

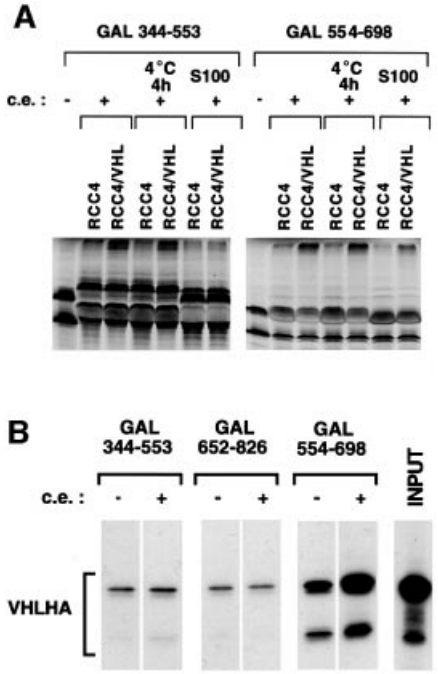

Fig. 7. Different requirements for targeting of N- and C-terminal sites in the HIF-1α ODDD by VHLE3. (**A**) Comparison of unfractionated and S100 cytoplasmic extract. [^{35}S]methionine-labelled GAL344–553 and GAL554–698 substrates were ubiquitylated in fresh cytoplasmic extract (+), cytoplasmic extract that had been left at 4°C for 4 h (4°C 4 h+) or the S100 supernatant of cytoplasmic extract (S100+) from RCC4 or RCC4/VHL cells. The S100 extract supported VHLE3-dependent ubiquitylation of GAL554–698 but not GAL344–553. (**B**) Interaction of GAL-HIF–1α with HA·VHL produced in reticulocyte lysate. [^{35}S]methionine-labelled C-terminal HA-tagged VHL (VHLHA) or N-terminal HA-tagged VHL (data not shown) and non-radiolabelled GAL–HIF-1α substrates were mixed and incubated either in buffer (–) or in cytoplasmic extract (+) prior to immunoprecipitation using anti-GAL. Co-immunoprecipitation of VHLHA occurred with GAL554–698, whilst background levels of binding were obtained with GAL652–826 and GAL344–553. Equivalent immunoprecipitation of the GAL–HIF-1α proteins was confirmed by anti-GAL immunoblotting (data not shown).

Pro402, whilst a more extensive polypeptide (e.g. residues 380–417) is necessary for efficient modification of the HIF-1α substrate by the hydroxylase.

The N- and C-terminal VHLE3 target sites differ in their functional requirements

The extensive ODDD in HIF-1α therefore contains two discrete interaction sites for VHLE3 that appear to be regulated in a similar manner by prolyl hydroxylation. Since previous analyses in this and other laboratories had identified the C- but not the N-terminal site, this suggested that there may also be differences in the mechanism of interaction at the two sites.

First, in a previous domain analysis of HIF-1α ubiquitylation, the N-terminal VHLE3 target site was not detected as a target (Ohh *et al.*, 2000). We considered whether this might have been due to the use of S100 rather than crude cytoplasmic extract for ubiquitylation assays in that analysis. VHLE3-dependent ubiquitylation of both the N- and C-terminal VHLE3 target sites was therefore compared using the standard cytoplasmic extract or S100 (Figure 7A). Consistent with the published data (Ohh *et al.*,

2000), we found that VHLE3-dependent ubiquitylation of the C-terminal VHLE3 target site (represented by GAL554–698) was observed using the S100 extract (Figure 7A). However, VHLE3-dependent ubiquitylation at the N-terminal VHLE3 target site (represented by GAL344–553) was barely visible in the S100 extract, suggesting that a factor required for ubiquitylation of the N-terminal VHLE3 target site is either lost or inactivated during S100 preparation.

Secondly, previous analyses of interactions between HIF-1α and pVHL identified only the C-terminal site (Cockman *et al*., 2000; Ohh *et al*., 2000; Tanimoto *et al*., 2000; Yu *et al*., 2001). These analyses used HIF-1α and pVHL prepared in reticulocyte lysate. In view of the above requirement for modification of the N-terminal ODDD by cytoplasmic extract in order to promote ubiquitylation, we tested whether exposure to cytoplasmic extract was sufficient to promote interaction of the N-terminal ODDD sequences with pVHL prepared in reticuloctye lysate. Results shown in Figure 7B show that this is not the case. The modified N-terminal ODDD could capture pVHL in 786-0 HA·VHL cell lysates but remained unable to bind pVHL produced by reticulocyte IVTT. This suggests that either other components of the VHLE3 complex, or a modification of pVHL, which occurs *in vivo* but not in reticulocyte IVTT, is necessary for the N-terminal interaction. Therefore, although both sites within the ODDD are capable of interaction with VHLE3, they differ importantly in their requirements for interaction.

Discussion

In this work, we demonstrate that HIF-1α contains two independent regions in the ODDD that are targeted by the VHLE3 ubiquitin ligase. *In vitro* assays demonstrated that both sites can function independently, supporting interactions with the VHLE3 complex and VHLE3-dependent ubiquitylation. Furthermore, mutational analysis demonstrated the functional importance of each site in regulating transcriptional responses *in vivo*.

At the C-terminal site, residues 556–572 previously have been defined as a minimal VHLE3 interaction domain (Tanimoto *et al*., 2000). The current study has defined a minimal VHLE3 target site within HIF-1α residues 380–417. The definition of this second site provides additional evidence for the critical role played by pVHL in the regulation of the HIF system, and also explains previously puzzling observations regarding the function of isolated portions of the ODDD. Whilst previous studies have indicated that interaction with the VHLE3 complex appears to be limited to the C-terminal site, both N- and C-terminal sequences within the ODDD are able to mediate partial levels of protein instability. This has been reported both in the context of the native HIF-1α molecule and when isolated subsequences from HIF-1α and HIF-2α have been analysed as fusions to heterologous proteins (Huang *et al*., 1998; Ema *et al*., 1999; O'Rourke *et al*., 1999; Sutter *et al*., 2000; Yu *et al*., 2001). The current work indicates that the observed partial instability may be accounted for by the operation of one, but not both VHLE3 target sites.

Importantly, both VHLE3 target sites in HIF-1α appear to be regulated by enzymatic hydroxylation of specific prolyl residues. Hydroxylation of Pro564 recently has been identified as the key modification controlling activity of the C-terminal target site (Ivan *et al*., 2001; Jaakkola *et al*., 2001). Analysis of the N-terminal VHLE3 interaction site now implicates hydroxylation of Pro402 in the regulation of targeting at this site, and demonstrates the presence of a common motif, LXXLAP, at the two sites. Thus, the new findings provide further evidence for the importance of prolyl hydroxylation in the regulation of HIF, and for the importance of prolyl hydroxylation as a mechanism of protein recognition by the VHLE3 complex.

However, despite the similarities in the operation of the two VHLE3 target sites, important differences were defined. First, reticulocyte lysate contains an activity that is able to hydroxylate the C-terminal site effectively and to promote interaction with the VHLE3 complex. In contrast, exposure to RCC4 or other tissue culture cell cytoplasmic extract was required to promote the VHLE3 interaction with the N-terminal site, whereas reticulocyte lysate had no such activity. Secondly, although both VHLE3 target sites contain a common motif, the sequences required for efficient enzymatic modification appear to extend well beyond this motif and are quite different at the two sites. For instance, the synthetic peptide B28HYP corresponding to HIF-1α residues 390–417 and containing hydroxyproline at residue 402 could bind to VHLE3 and therefore must contain the VHLE3 interaction determinants. Nevertheless, neither the corresponding peptide containing proline, B28PRO, nor the GAL-HIF-1α fusion GAL390–417, could be modified by cytoplasmic extract so as to capture VHLE3, indicating that more extensive sequences were required to direct the modification step. Taken together, these findings suggest either that additional factors present in RCC4 cytoplasmic extract but not reticulocyte extract are required for sequence recognition and hydroxylation at the N-terminal site, or that a different enzyme or enzyme isoform is involved.

The HIF-1α sites also differed in their mode of interaction with the VHLE3 complex. Whereas the hydroxylated C-terminal site interacts readily with recombinant pVHL produced in a variety of expression systems, and has been shown to interact directly with pVHL, the N-terminal site could not interact with recombinant pVHL expressed *in vitro* in reticulocyte lysate. In contrast, a robust interaction of the N-terminal target site was obtained with VHLE3 derived from lysates of 786-0 HA·VHL cells. This difference could indicate the need for a modification of pVHL that occurs *in vivo* but not *in vitro* in reticulocyte lysate, or the operation of an additional factor that cooperates in a more complex interaction with VHLE3 at the N-terminal target site. So far, however, we have not been able to reconstitute the interaction *in vitro* with known components of this complex.

Whatever the precise reasons for these findings, the existence of more than one destruction domain targeted by VHLE3 is itself of interest. Similar analyses in other systems have indicated that multiple destruction domains are not uncommon among proteins that are regulated by degradation. For instance, there are several examples of cell cycle proteins that contain two destruction boxes (D boxes) targeted by the anaphase-promoting complex,

another multicomponent E3 ligase. In these cases, the D boxes also differ in overall sequence and potency in some assays. The reasons why these proteins have evolved multiple destruction boxes are not well understood, but they presumably provide for increased combinatorial interactions that lend specificity to the destruction process.

Modification of the destruction domains in HIF-α subunits by enzymatic prolyl hydroxylation is of particular interest in relation to the underlying physiology of oxygen sensing. Recent evidence has indicated that hydroxylation at Pro564 is performed by one or more members of the 2-oxoglutarate-dependent dioxygenase superfamily (Jaakkola *et al*., 2001). The use of molecular oxygen as co-substrate by such enzymes provides a direct link between HIF regulation and the availability of molecular oxygen. However, it is difficult to envisage how modification of a particular peptide substrate by a single enzyme could account for the precisely shaped physiological responses of the system. Many other systems of oxygen sensing have been proposed and might impact at different points in the pathway (Semenza, 1999; Zhu and Bunn, 2001). Equally, other ubiquitin ligase systems such as mdm-2 have been proposed to impact on HIF regulation (Ravi *et al*., 2000), although the site and mode of targeting of the HIF system have not been defined, and might involve different oxygen-sensitive processes. Nevertheless, the recognition of two sites of modification by prolyl hydroxylation with different properties with respect to the modifying activity and VHLE3 interaction indicates a potential for more complex responses to oxygen availability to be mediated through enzymatic prolyl hydroxylation. It will now be of interest to identify the prolyl hydroxylase(s) operating at the different VHLE3 target sites and compare their oxygen-dependent characteristics for modification of different degradation domains.

Materials and methods

Plasmids

His_6-tagged mouse E1 cDNA in pRSET was kindly donated by T.Hunt. GAL fusion proteins were encoded by plasmids based on pcDNA4 that contain a truncated GAL4 gene encoding amino acids 1–147 followed by a polylinker bearing *Sac*II and *Asc*I sites into which the HIF-1α or HIF-2α sequences generated by PCR were cloned. All PCRs were performed using *Pfu* DNA polymerase (Stratagene). pcDNA3-HIF-1α, pVHLHA, pGAL/VP16, pGAL/α344–417/VP16, pUAS-tk-Luc and pCMVβGal have been described previously (Pugh *et al*., 1997; O'Rourke *et al*., 1999; Cockman *et al*., 2000). pGL3PGK6TKp contained six copies of the HRE from the mouse phosphoglycerate kinase-1 gene linked to a luciferase reporter gene. pGAL/344–400/VP16 was constructed by insertion of HIF-1α sequence encoding amino acids 344–400 into *Sac*II–*Asc*I-digested pGAL/VP16. Mutations were generated using a site-directed mutagenesis kit (QuickChange; Stratagene) and mutagenic oligonucleotides designed according to the manufacturer's recommendations. The integrity of all plasmids was confirmed by DNA sequencing.

Cell culture and transient transfection

RCC4 cells stably transfected with pcDNA3-VHL (RCC4/VHL) or empty vector (RCC4) (Cockman *et al*., 2000) and the HIF-1α-deficient CHO cell line (Ka13) (Wood *et al*., 1998) have been described previously. 786-0 HA·VHL cells (786-0 cells stably transfected with plasmid pRC-HA-VHL) were a gift from W.G.Kaelin, and U2OS cells were a gift from S.Geley. All cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, glutamine (2 mM), penicillin (50 IU/ml) and streptomycin sulfate (50 μg/ml). For RCC4, RCC4VHL and 786-0 HA·VHL, G418 (0.5 mg/ml) was added to the growth medium.

Transient transfections were performed using Fugene 6 (Roche Molecular Biochemicals). For luciferase assay in U20S cells, 10 ng of GAL/HIF-1α/VP16 activator plasmid, 100 ng of pUAS-tk-Luc luciferase reporter and 500 ng of pCMVβgal (to enable correction for variation in transfection efficiency) were used per well of a 6-well plate. For luciferase assay in Ka13 cells, 2 μg of pcDNA3-HIF-1α plasmid, 0.1 μg of pGL3PGK6TKp luciferase reporter and 0.5 μg of pCMVβgal were used. Hypoxic incubation was in an atmosphere of 0.1% oxygen, 5% CO_2, balance nitrogen in a Napco 7001 incubator (Jouan). For immunoblotting experiments, Ka13 cells were transfected with 2 μg of pcDNA3-HIF-1α wild-type and mutant plasmids.

Luciferase and β-galactosidase assays

Luciferase activities were determined in extracts made from transfected cells maintained for 48 h, either entirely in normoxia or with hypoxic stimulation for the final 16 h. Luciferase activities were determined using a commercially available luciferase assay system (Promega) and a TD-20e luminometer (Turner Designs). Relative β-galactosidase activity in extracts was measured using *o*-nitrophenyl-β-D-galactopyranoside (0.67 mg/ml) as substrate in a 0.1 M phosphate buffer pH 7.0 containing 10 mM KCl, 1 mM $MgSO_4$ and 30 mM β-mercaptoethanol incubated at 30°C for 15–45 min. The A_{420} was determined after stopping the reaction by the addition of sodium carbonate to a final concentration of 0.4 M.

Substrate and cell extract preparation

[^{35}S]methionine-labelled HIF-1α and GAL-HIF-1α substrates were prepared by coupled IVTT using TnT7 rabbit reticulocyte (Promega). GAL–HIF-1α proteins appear as two bands, with the faster migrating form arising from aberrant translational initiation within the GAL sequence. In all assays, the faster migrating form is recognized less efficiently by VHLE3.

Ka13 whole-cell extracts for immunoblotting were prepared 24 h after transfection by lysis in 8 M urea, 10% glycerol, 1% SDS, 5 mM dithiothreitol (DTT), 10 mM Tris pH 6.8, followed by disruption using a hand-held homogenizer (Ultra-Turrax T8 with 5G dispersing tool; Janke & Kunkel GmbH). Cytoplasmic extract for ubiquitylation assays and for modification of HIF-1α substrates *in vitro* was prepared as previously described (Cockman *et al*., 2000). Briefly, cells were washed twice with cold hypotonic extraction buffer (HEB: 20 mM Tris pH 7.5, 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT). After removal of excess buffer, cells were lysed in a Dounce homogenizer. Following lysis, crude cytoplasmic extract was centrifuged at 10 000 *g* for 5 min at 4°C to remove cell debris and nuclei, and stored in aliquots at –70°C. Heat-treated cytoplasmic extract was prepared by incubation at 60°C for 3 min, followed by centrifugation at 10 000 *g* for 5 min to remove precipitated material. S100 extract was obtained by an additional ultracentrifugation step at 100 000 *g* at 4°C for 4 h. Cytoplasmic extract was also incubated at 4°C for 4 h to ensure that effects seen with S100 extract were due to the 100 000 *g* spin. ATP-depleted cytoplasmic extract was prepared by hexokinase/glucose treatment as follows. To each 100 μl of cytoplasmic extract, 5 μl of 1 M glucose and 5 U of 1 U/μl hexokinase (Sigma) were added and the sample incubated at 30°C for 30 min to allow ATP depletion. Nuclear extract was obtained by extraction of the crude nuclear pellet that remains following cytoplasmic extract preparation with three volumes of buffer C [20 mM Tris pH 8.0, 25% glycerol (v/v), 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA], followed by 3-fold dilution in 20 mM Tris pH 8.0 and storage at –70°C. 786-0 HA·VHL cell extract, used as a source of VHLE3, was prepared by lysis in NP-40 lysis buffer (10 mM Tris pH 7.5, 0.25 M NaCl, 0.5% NP-40). NP-40 lysis buffer does not support the HIF-1α-modifying activity (data not shown).

Modification of substrate was achieved by incubation of GAL–HIF-1α translate (4 μl) with 70 μl of RCC4 cell lysate in HEB, or control HEB alone, at 30°C for 20 min prior to anti-GAL immunoprecipitation. For modification in the presence of Fe (II), ferrous chloride (100 μM) was added to the reaction. For modification in the presence of prolyl hydroxylase inhibitor, *N*-oxalylglycine (1 mM) was used either alone or in combination with 2-oxoglutarate (5 mM). Modification reactions using hexokinase-treated cytoplasmic extract and nuclear extract were as above.

Antibodies, immunoblotting and peptides

Mouse anti-HA antibody (12CA5) used for immunoprecipitation and rat anti-HA antibody (3F10) used for immunoblotting were from Roche Molecular Biochemicals. Anti-GAL4 (RK5C1) agarose conjugate was from Santa Cruz Biotechnology. Anti-HIF-1α antibody (clone54) was from Transduction Laboratories. Following SDS–PAGE, proteins were transferred on to Immobilon-P membrane (Millipore) and processed for

immunoblotting using the indicated antibody. Biotinylated HIF-1α peptides (Biopeptide Co.) were retrieved using streptavidin Dynabeads M-280 (Dynal ASA).

Ubiquitylation enzymes and assays

The E1-activating enzyme used in ubiquitylation assays was either obtained from Affiniti Research or purified from BL21 (DE3) *Escherichia coli* transfected with plasmid expressing His_6-tagged mouse E1. His_6-E1 was purified by Ni^{2+}–agarose affinity chromatography. After dialysis against phosphate-buffered saline, glycerol was added to 10% (v/v) and 25 ng/μl aliquots stored at –80°C. Human CDC34 recombinant E2 enzyme was from Affiniti Research. VHLE3 was obtained by anti-HA immunoprecipitation from stably transfected 786-0 HA·VHL cell lysates. Briefly, 1 ml of 786-0 HA·VHL cell lysate (~10^7 cells) and 5 μg of anti-HA antibody were incubated at 4°C for 1 h. A 12 μl aliquot of protein G–Sepharose beads was added and incubation continued at 4°C with mixing. Beads were then washed four times in IP wash buffer (125 mM NaCl, 25 mM Tris pH 7.5, 0.1% NP-40), with a final wash in HEB. GAL–HIF-1α substrate was prepared by anti-GAL immunoprecipitation from [^{35}S]methionine-labelled TnT7 rabbit reticulocyte (Promega) translates.

The purified component ubiquitylation reaction (40 μl) consisted of 4 μl of 5 mg/ml ubiquitin, 4 μl of 10× ATP-regenerating system (20 mM Tris pH 7.5, 10 mM ATP, 10 mM magnesium acetate, 300 mM creatine phosphate, 0.5 mg/ml creatine phosphokinase), 2 μl of E1, 3 μl of E2, 6 μl of VHLE3 immunopurified on protein G–Sepharose (~5×10^6 cells), 6 μl of GAL-HIF-1α substrate immunopurified on agarose beads (derived from 4 μl of translate) and 15 μl of HEB. Reactions were incubated at 30°C for 2 h with occasional mixing, stopped by the addition of SDS sample buffer and analysed by SDS–PAGE and autoradiography. For ubiquitylation reactions in the presence of peptide, peptides were added at a final concentration of 18.5 μM.

The cytoplasmic extract-based ubiquitylation assays have been described previously (Cockman *et al*., 2000).

In vitro interaction assays

For pVHL interaction assays, HEB-treated or cytoplasmic extract-modified GAL–HIF-1α substrates (4 μl, non-radiolabelled) were immunopurified using anti-GAL antibody-conjugated Sepharose beads (10 μl of beads). [^{35}S]methionine-labelled VHLHA translate (4 μl) was added together with 60 μl of HEB and samples incubated at 30°C for 1 h with mixing. Beads were then washed four times with IP wash buffer and once with IP wash buffer lacking NP-40. Co-precipitating VHLHA proteins were analysed by SDS–PAGE and fluorography.

For VHLE3 interaction assays, cytoplasmic extract-modified GAL–HIF-1α substrates were incubated together with 786-0 HA·VHL cell lysate and anti-HA antibody (2.5 μg) at 4°C for 1 h. Protein G–Sepharose beads were added and samples mixed at 4°C for 30 min. Beads were washed as above. Co-precipitating GAL–HIF-1α proteins were analysed by SDS–PAGE and fluorography.

For VHLE3 capture assays using biotinylated peptides, HEB- or cytoplasmic extract-treated peptides (750 pmol) were incubated with 786-0 HA·VHL cell lysate at 4°C for 1 h. Streptavidin dynabeads (7×10^7 beads) were added and samples mixed at 4°C for a further 30 min. Beads were washed four times with IP wash buffer and once with IP wash buffer lacking NP-40. Co-precipitating HA·VHL was analysed by anti-HA immunoblotting.

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